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An Autoimmune Disease-Associated CTLA-4 Splice Variant Lacking the B7 Binding Domain Signals Negatively in T Cells

Lalitha Vijayakrishnan,[1] Jacqueline M. Slavik,[1,6] Zsolt Illés,[1,6] Rebecca J. Greenwald,[2] Dan Rainbow,[3] Bernhard Greve,[1] Laurence B. Peterson,[4] David A. Hafler,[1] Gordon J. Freeman,[5] Arlene H. Sharpe,[2] Linda S. Wicker,[3,*] and Vijay K. Kuchroo[1,*]

[1]Center for Neurologic Diseases
Department of Neurology
[2]Immunology Research Division
Department of Pathology
Brigham and Women's Hospital and
Harvard Medical School
Boston, Massachusetts 02115
[3]Juvenile Diabetes Research Foundation/
Wellcome Trust
Diabetes and Inflammation Laboratory
Cambridge Institute for Medical Research
University of Cambridge
Wellcome Trust/MRC Building
Cambridge, CB2 2XY
United Kingdom
[4]Department of Pharmacology
Merck Research Laboratories
Rahway, New Jersey 07065
[5]Department of Medical Oncology
Dana-Farber Cancer Institute
Department of Medicine
Harvard Medical School
Boston, Massachusetts 02115

Summary

Cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) plays a critical role in downregulating T cell responses. A number of autoimmune diseases have shown genetic linkage to the CTLA-4 locus. We have cloned and expressed an alternatively spliced form of CTLA-4 that has genetic linkage with type I diabetes in the NOD mice. This splice variant of CTLA-4, named ligand-independent CTLA-4 (liCTLA-4), lacks exon2 including the MYPPPY motif essential for binding to the costimulatory ligands B7-1 and B7-2. Here we show that liCTLA-4 is expressed as a protein in primary T cells and strongly inhibits T cell responses by binding and dephosphorylating the TcRζ chain. Expression of liCTLA-4, but not full-length CTLA-4 (flCTLA-4), was higher in memory/regulatory T cells from diabetes-resistant NOD congenic mice compared to susceptible NOD mice. These data suggest that increased expression and negative signaling delivered by the liCTLA-4 may regulate development of T cell-mediated autoimmune diseases.

*Correspondence: vkuchroo@rics.bwh.harvard.edu (V.K.K.), linda.wicker@cimr.cam.ac.uk (L.S.W.)
[6]These authors contributed equally to this work.

Introduction

CTLA-4 is a critical downregulatory molecule expressed on T cells that plays a major role in inhibiting T cell activation and peripheral tolerance (Greenwald et al., 2002). Its predominant role in suppressing T cell function is best reflected in the phenotype of CTLA-4$^{-/-}$ mice, which rapidly develop lymphoproliferative disease with multiorgan lymphocyte infiltration and tissue destruction (Brunner et al., 1999; Chambers et al., 1997; Krummel and Allison, 1996). CTLA-4 is located on mouse chromosome 1 within a genetic interval, which also contains other major T cell costimulatory molecules, CD28 and ICOS. By congenic mapping it was established that this locus (defined as *Idd5.1*) containing the costimulatory genes CD28, CTLA-4, and ICOS, when replaced with a genetic interval from autoimmune-resistant B10 strain, could confer resistance to diabetes in the NOD mice (Colucci et al., 1997; Hill et al., 2000; Lamhamedi-Cherradi et al., 2001). The region is syntenic with human chromosome 2q33, and autoimmune diseases such as Multiple Sclerosis (Sawcer et al., 1996), IDDM (Copeman et al., 1995), Graves' disease (Kouki et al., 2002), Hashimoto's thyroiditis (Nithiyananthan et al., 2002), Addison's disease, and Rheumatoid Arthritis (Becker et al., 1998) have been shown to be associated with the locus.

In spite of all the genetic linkage and functional data on CTLA-4, no major polymorphisms in CTLA-4 gene have been identified in mice or humans. In a recent study, splice variants of CTLA-4 have been identified as candidates for risk of Grave's disease, Autoimmune hypothyroidism, and Type 1 Diabetes (Ueda et al., 2003). In humans, disease susceptibility mapped to an allelic variation in the noncoding 6.1 kb 3′ region of CTLA-4 and correlated with mRNA levels of the soluble CTLA-4. However, in the mouse model of Type 1 Diabetes, increased disease risk was correlated with differential mRNA levels of a novel splice variant named liCTLA-4, which lacked the Exon2 encoding the CD80/CD86 ligand binding domain. This raised an important issue as to whether the liCTLA-4, which lacks the CD80/CD86 ligand binding domain of full- length CTLA-4 (flCTLA-4), is expressed as a functional protein and can modulate T cell responses. Furthermore, does subtle variation in the expression level of liCTLA-4 in diabetes susceptible and resistant strains of mice alter T cell activation?

In this paper, we demonstrate that liCTLA-4 is expressed as a protein in normal peripheral T cells. We show that in spite of the loss of an extracellular ligand binding domain, when the liCTLA-4 was expressed in CTLA-4$^{-/-}$ T cells, the liCTLA-4 was more potent than the flCTLA-4 in inhibiting T cell responses, inhibiting both T cell proliferation and cytokine secretion. Further, in support of its genetic relevance, we show that there is an enhanced expression of the liCTLA-4 in memory T cells of a diabetes-resistant NOD congenic strain resulting in decreased TcRζ phosphorylation and lack of ZAP70 activity in these cells. Our data suggest that the liCTLA-4 may regulate activation of memory/effector

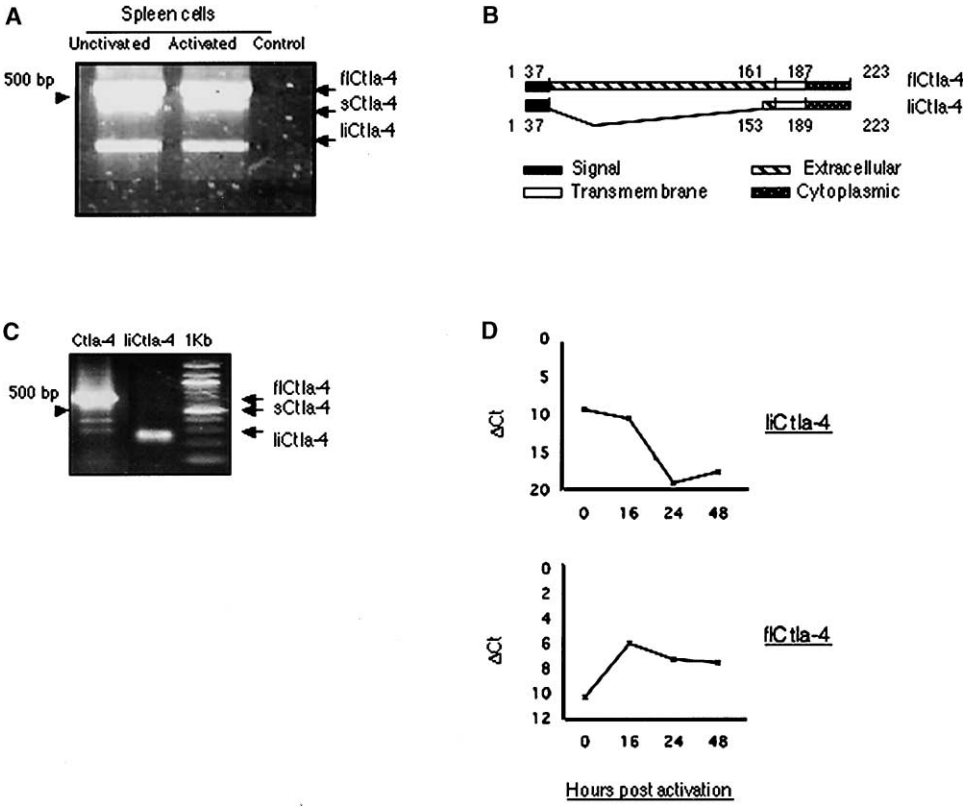

Figure 1. mRNA Expression of liCTLA-4 in T Cells

(A) Agarose gel showing spliced isoforms of CTLA-4 generated by RT-PCR. mRNA was isolated from unactivated or anti-CD3 activated purified spleen cells of C57Bl/6 mice. RT-PCR products from unactivated (lane1) or activated (lane 2) spleen cells using primers designed to amplify flCTLA-4 are shown. Lane 3 indicates the water control.

(B) Schematic showing comparative protein domains of flCTLA-4 and liCTLA-4. The protein structure of flCTLA-4 is compared to the predicted structure and protein domains of liCTLA-4. The predicted amino acid sequence of liCTLA-4 was found to lack the IgV domain (residues 38-161) and the B7 interacting MYPPPY motif .

(C) Agarose gel showing RT-PCR amplification of liCTLA-4 using a junctional primer in exon1/3 of liCTLA-4. mRNA was isolated from anti-CD3 activated purified spleen cells of C57Bl/6 mice. Spliced isoforms of CTLA-4 were generated by RT-PCR amplification from total mRNA using primers in exon1 and exon4 of flCTLA-4 (lane 1) and primers in exon1/3 and exon 4 of liCTLA-4 (lane 2); lane 3 is loaded with a 1 Kb ladder.

(D) Comparative expression kinetics in mRNA of flCTLA-4 and liCTLA-4 in resting and activated C57Bl/6 T cells. CD3 + ve T cells enriched from C57Bl/6 were stimulated with anti-CD3 and anti-CD28 as described in the Experimental Procedures. Relative expression of flCTLA-4 (a) and liCTLA-4 (b) mRNA was determined by real-time (TaqMan) RT-PCR in cultured T cells at the indicated time points (hours). Representative data of three separate experiments are shown.

T cells and thus play a role in preventing susceptibility to T cell-mediated autoimmune diseases.

Results

By RT-PCR, three different forms of CTLA-4 (CD152) were detected in unactivated and activated spleen cells of C57Bl/6 mice: (1) a full-length form of CTLA-4, (flCTLA-4); (2) a soluble CTLA-4, (sCTLA-4); and (3) a splice variant of approximately 300 bp (Figure 1A, lanes 1 and 2). Sequencing the RT-PCR products showed that, unlike the flCTLA-4 and the sCTLA-4, the splice variant (liCTLA-4) lacked exon 2 encoding the extracellular IgV-like domain of flCTLA-4. The predicted amino acid sequence of liCTLA-4 (108 residues) contained a signal sequence, a transmembrane domain and cytoplasmic domain similar to the flCTLA-4 (Figure 1B), but lacked the IgV exon encoding the B7 (CD80 and CD86) binding MYPPPY motif. The mature cDNA of liCTLA-4 isoform is predicted to be of a smaller size (about 8 kDa) than the flCLTA-4 isoform because of the loss of residues 37-153, including glycosylation sites (Figure 1B). To confirm that the mRNA for liCTLA-4 is present in T cells, the sequencing data obtained above was used to develop a forward primer at the exon1/3 junction and a reverse primer in exon4 to specifically amplify liCTLA-4, and not flCTLA-4 or sCTLA-4. This primer pair specifically amplified a single product of ~200 bp, which upon sequencing was found to be consistent with the sequence of liCTLA-4 (Figure 1C, lane 2).

Previous studies have shown that flCTLA-4 is not expressed on the surface of resting T cells but rather is induced following crosslinking of CD3 and CD28 on both CD4 and CD8 T cells. We compared the expression kinetics of liCTLA-4 with that of flCTLA-4 in T cells by designing primers and probes to specifically detect either of the isoforms in a real-time RT-PCR. Consistent with previous studies (Lindsten et al., 1993), in T cells

activated with antibodies to CD3 and CD28 flCTLA-4 gene expression was rapidly induced within 16 hr and plateaued 24 hr postactivation (Figure 1D). Interestingly, maximal levels of liCTLA-4 were seen in unactivated T cells, and following activation there was a 3- to 4-fold decrease in liCTLA-4 mRNA, compared to its baseline levels. However, 48 hr postactivation the levels of liCTLA-4 mRNA appeared to be reinduced. Thus, while the expression of flCTLA-4 is activation dependent, in contrast, the expression of liCTLA-4 appeared to be constitutive. Further, during early stages of T cell activation, the mRNA levels of liCTLA-4 and flCTLA-4 followed reciprocal pattern expression kinetics but both forms were expressed during late T cell activation.

To determine if liCTLA-4 is expressed as a protein, cDNA of liCTLA-4 was obtained by gel extraction and purification of the lower 300 bp band seen in Figure 1A, lane 1. The purified cDNA was cloned in a mammalian expression vector, pCMV-TAG5C. HEK293 cells transfected with pCMV-TAG5C encoding liCTLA-4 expressed a protein of approximately 8 kDa on a reducing gel detected by antibodies to the MYC tag by Western blot (Figure 2A). Since liCTLA-4 lacked most of the extracellular domain, we tested whether the protein is targeted to the cell membrane or trapped and retained in the cytosol. The liCTLA-4 cDNA cloned in pCMV-TAG-2C was modified to include a 10 amino acid MYC tag between the putative signal sequence and the amino terminus of the extracellular domain between amino acid positions Asp154 and Pro155. HEK293 cells transfected with the construct were stained with anti-MYC-FITC antibody and analyzed by flow cytometry for surface expression of liCTLA-4. Figure 2B shows MYC-liCTLA-4 expression 48 hr posttransfection on the HEK293 cell surface consistent with the liCTLA-4 expression on the cell surface. An 11 amino acid residue, including the YVKM motif, in the cytoplasmic domain of flCTLA-4 interacts with AP-50 of the clathrin-associated coated pit adaptor protein complex AP2 that is essential for intracellular localization and trafficking of flCTLA-4. Since both the liCTLA-4 and flCTLA-4 share identical amino acid sequence in the cytoplasmic domain, we wished to compare their localization pattern in mammalian cells. liCTLA-4, flCTLA-4, and flCTLA-4 lacking its cytoplasmic domain (flCTLA-4 tailless) was cloned into flourescent tagged mammalian expression vectors, and these modified vectors were used to transfect HEK293T cells. Whereas flCTLA-4 tailless was found to be distributed evenly throughout the cytosol (Figure 2Cii), liCTLA-4 was found to be distributed in intracellular vesicles within the cytosol (Figure 2Ci). This pattern of localization was similar to flCTLA-4, since in cotransfections, both liCTLA-4 and flCTLA-4 followed an identical pattern of localization (Figures 2Ciii and 2Civ). Together these data show that mRNA of liCTLA-4 can be translated to express a protein of ~8 kDa, and even though the liCTLA-4 lacks a major portion of the extracellular domain, it localizes within intracellular vesicles in the cytosol and also translocates to the cell surface in mammalian cells.

To determine whether liCTLA-4 is expressed in normal T cells, activated spleen cell lysates from C57/BL6 splenocytes were subjected to Western blotting using an antibody (C-19) directed to the cytoplasmic tail of CTLA-4. This anti-CTLA-4 antibody was able to detect three bands on a Western blot. Besides the doublet of flCTLA-4 migrating at ~34 kDa, the antibody was able to detect a single band at 8 kDa consistent with it being the liCTLA-4 (Figure 2D). As described previously (Lee et al., 1998) in a 2D gel analysis, the flCTLA-4 protein generally appears as a doublet migrating at 34 kDa that may represent differentially glycosylated forms of flCTLA-4. The flCTLA-4 molecule has three potential N-linked glycosylation sites while no predicted N-linked sites are present in liCTLA-4. Cell lysates from activated spleen cells of CTLA-4$^{-/-}$ mice did not show any band consistent with flCTLA-4 or liCTLA-4.

The flCTLA-4 is not detected in naive T cells but is rapidly upregulated upon T cell activation with maximal expression observed after 48 hr of activation. To study the protein expression kinetics of liCTLA-4, T cells from splenocytes of DO11.10/Rag$^{-/-}$ TCR transgenic mice were activated with cognate antigen (Ovalbumin) in the presence of syngenic APCs. Cells were then analyzed for expression of either flCTLA-4 or liCTLA-4 by Western blotting at various time points over 48 hr using the antibody directed to the tail of CTLA-4. The T cells ex vivo, without further in vitro activation, had a strong signal for the liCTLA-4 but not for the flCTLA-4. These cells are a mixture of both activated/memory T cells and naive T cells. Thus, it is not clear whether the liCTLA-4 expressed in these resting cells is on naive or memory T cells. In T cells activated with specific antigen, the level of liCTLA-4 protein appeared to go down at 16 and 24 hr postactivation, while flCTLA-4 began to appear. However, at 48 hr postactivation, both liCTLA-4 and flCTLA-4 were detectable (Figure 2E). Thus, consistent with the mRNA expression kinetics, the liCTLA-4 protein was expressed in resting T cells but rapidly downregulated early during T cell activation. However, in agreement with previous studies (Lindsten et al., 1993), the expression of flCTLA-4 was low in resting T cells and was rapidly upregulated upon activation. This reciprocal regulation of liCTLA-4 and flCTLA-4 expression early during T cell activation suggests a differential requirement for these molecules in T cells. Though it is known that the flCTLA-4 downregulates T cell function by regulating proximal TCR signaling events, the function of liCTLA-4 is not known and forms the focus of this study.

flCTLA-4 signaling has been shown to prevent cell cycle entry at the G1 phase and subsequently to inhibit proliferation and IL-2 production (Krummel and Allison, 1996; Walunas et al., 1994). To investigate the function of liCTLA-4 in T cells in comparison to flCTLA-4, we expressed flCTLA-4 or liCTLA-4 by retroviral infection (Costa et al., 2000) in T cells from triple knockout (TKO) mice that lack CTLA-4, B7.1, and B7.2 (Mandelbrot et al., 1999). The retroviral vector has a multiple cloning site allowing incorporation of genes expressing the test protein and a downstream selectable marker, GFP (Costa et al., 2000). Using T cells from TKO mice was crucial for analysis of these experiments, since the TKO mice lack endogenous isoforms of either flCTLA-4, sCTLA-4, or liCTLA-4 that may otherwise interfere with the functional activity of exogenously introduced liCTLA-4 or flCTLA-4. Furthermore, because both B7 molecules are absent in TKO cells, TKO mice provide a source of naive T cells as compared to those in

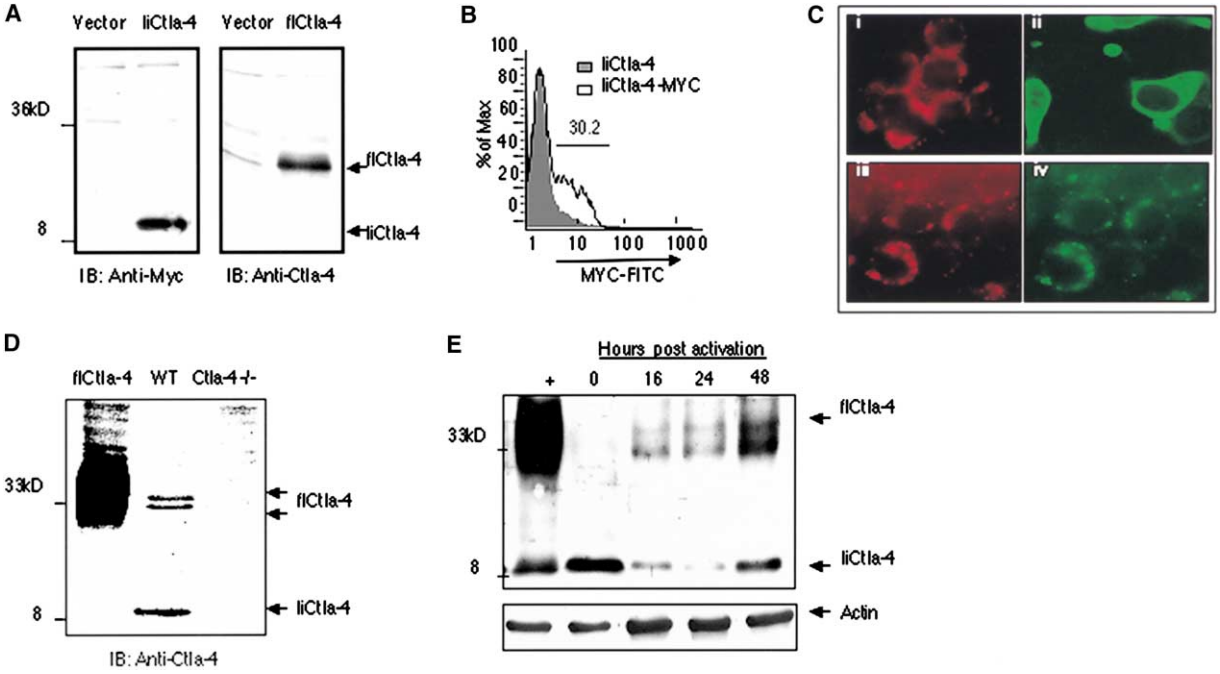

Figure 2. Protein Expression of flCTLA-4 and liCTLA-4 in T Cells

(A) Western blot showing the expression flCTLA-4 and liCTLA-4 cDNA in HEK293T cells. HEK 293T cells were transiently transfected with liCTLA-4 expressed in pCMV-TAG5C as a MYC tagged protein and flCTLA-4 expressed in pCMV-HA. Cell lysates from cells expressing control vector only or vector encoding liCTLA-4 or flCTLA-4 were analyzed by Western blotting. Lane 1, pCMV-TAG5C; lane 2, pCMV-Tag5C encoding liCTLA-4; lane 3, pCMV-HA; lane 4, pCMV-HA encoding flCTLA-4. Lanes 1 and 2 represent an immunoblot developed using an anti-MYC antibody and lanes 3 and 4 represent an immunoblot developed using an anti-CTLA-4 antibody.

(B) Flow cytometric analysis showing the detection of liCTLA-4 on the cell surface of HEK 293T cells. The cDNA of liCTLA-4 cloned in pCMV-TAG2C was modified to contain a 10 amino acid MYC domain between the residues Asp154 and Pro155 in the amino terminus of the extracellular domain of liCTLA-4. HEK293T cells were transfected with the modified construct. Transfectants were then stained for cell surface expression of liCTLA-4-MYC by using an anti-MYC-FITC antibody and analyzed by flow cytometry. Vector containing the liCTLA-4 without the MYC insertion was used as control (shaded histogram).

(C) Localization of liCTLA-4, flCTLA-4, and flCTLA-4 tailless in mammalian cells. Images of HEK293T cells transfected with either liCTLA-4 expressed in pDsRedExpress-N1 (i), or flCTLA-4 tailless expressed in pEGFPN3 (ii), and coexpression of liCTLA-4 expressed in pDsRedExpress-N1, and flCTLA-4 expressed in pEGFPN3 (iii and iv). The images show liCTLA-4 in red and both flCTLA-4 and flCTLA-4 tailless in green. Images were acquired using a 63× objective.

(D) Western blot analysis showing protein expression of flCTLA-4 and liCTLA-4 in activated T cells. Spleen cells from C57BL/6 mice or C57BL/6/TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice were activated in vitro with soluble anti-CD3 (1 μg/ml) for 16 hr and lysed. Cell lysates were immunoblotted with an anti-CTLA-4 antibody (C-19, Santa Cruz), which recognizes the cytoplasmic domain of both flCTLA-4 and liCTLA-4. The blot shows lane 1, a positive control of lysates from HEK293T cells expressing flCTLA-4; lane 2, activated whole spleen cell lysates of C57BL/6 mice; and lane 3, a negative control of activated whole spleen cell lysates of C57BL/6/ TKO mice (B7.1, B7.2, CTLA-4$^{-/-}$).

(E) Western blot showing protein expression kinetics of flCTLA-4 and liCTLA-4 in resting and activated D011.10/Rag2$^{-/-}$ T cells. Spleen cells from D011.10 /Rag2$^{-/-}$ mice were activated with ovalbumin peptide (323-339) at 1 μg/ml for the indicated time points. 6 × 10^6 activated T cells were lysed and immunoblotted using anti-CTLA-4 antibody (C-19, Santa Cruz). Lane 1 includes as a positive control recombinant flCTLA-4 and liCTLA-4 coexpressed in HEK 293T cells. Figures represent data of three or more independent experiments.

CTLA-4$^{-/-}$ mice that have an activated phenotype. T cells from double knockout (DKO) mice, which lack B7.1 and B7.2 (Mandelbrot et al., 1999) but express CTLA-4, were infected with empty retrovirus and used as positive controls for endogenous CTLA-4 expression and function.

Infected CD3$^+$ T cells were monitored for GFP expression (used as a surrogate marker for expression levels of either flCTLA-4 or liCTLA-4 protein) by flow cytometry as shown in Figure 3A and sorted based on GFP expression. Surface expression of flCTLA-4 could be confirmed with an anti-CTLA-4 antibody, 4F10, by surface staining of TKO T cells infected with retrovirus expressing flCTLA-4 (Figure 3A, right panel). However, surface expression of liCTLA-4 could not be confirmed by flow cytometry because of the lack of an appropriate antibody capable of detecting only liCTLA-4. Therefore, expression of liCTLA-4 and flCTLA-4 protein in the GFP-positive cells was confirmed by Western blot analysis (Figure 3B). The GFP-positive CD4 T cells were sorted, and sorted T cells expressing flCTLA-4 or liCTLA-4 (verified by both FACS analysis and Western blotting) were tested for functional activity by in vitro activation with varying concentrations of soluble anti-CD3 in the presence of mitomycin-treated antigen-presenting cells from wild-type BALB/c mice. As expected, sorted GFP-positive DKO T cells infected with empty retrovirus or the sorted GFP-positive TKO T cells infected with retrovirus encoding flCTLA-4 showed lower proliferation and IFNγ production when compared to sorted GFP-positive TKO T cells infected with empty retrovirus. Surprisingly, sorted GFP-positive TKO T cells infected with retrovirus

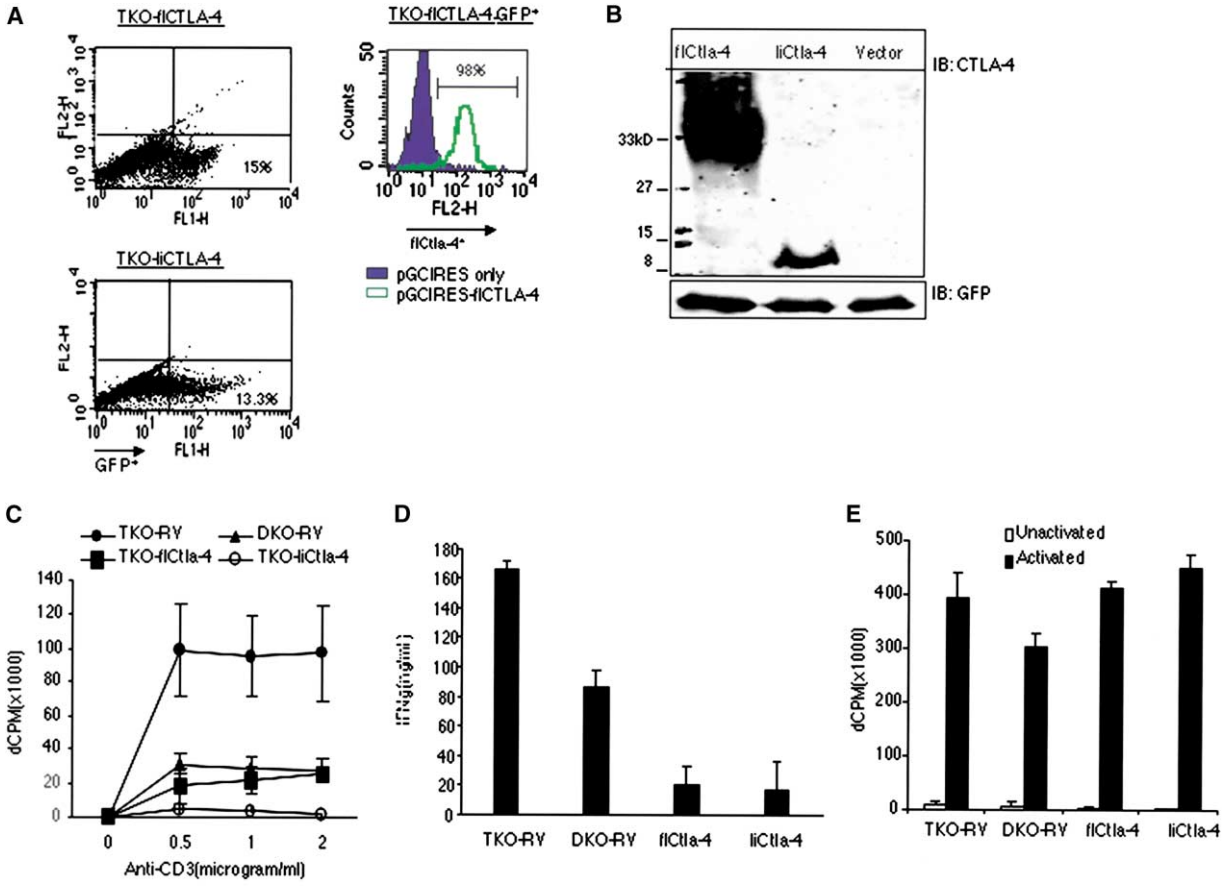

Figure 3. Functional Analysis of liCTLA-4

Activated CD3$^+$ T cells from TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice were infected with retrovirus expressing either the flCTLA-4, liCTLA-4, or empty retroviral vector (RV) as described in the Experimental Procedures. Infected GFP$^+$ cells were stimulated with mitomycin-treated APCs from normal BALB/c mice in the presence of varying concentrations of anti-CD3.

(A) Flow cytometric analysis of retrovirus-infected TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells. Panels on the left show comparable levels of GFP expression in the TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells infected with flCTLA-4 and liCTLA-4. Sorted GFP positive cells transfected with flCTLA-4 were stained with anti-CTLA-4 antibodies to confirm the expression of flCTLA-4 (right panel). Filled histogram indicates T cells infected with pGCIRES vector only and the line histogram indicates flCTLA-4 expression in TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells infected with pGCIRES expressing flCTLA-4.

(B) Western blot analysis of cell lysates prepared from retrovirus-infected TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells for the expression of flCTLA-4 and liCTLA-4. Western blot was performed as described in the Experimental Procedures. CTLA-4 isoforms were detected in infected TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells by an anti-CTLA-4 antibody recognizing the cytoplasmic domain of flCTLA-4 and liCTLA-4. TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells expressing flCTLA-4, lane 1; liCTLA-4, lane 2; and empty retroviral vector (RV), lane 3. Lower panel shows the loading control for the detection of equal amounts of GFP in the same lysates using an anti-GFP antibody.

(C and D) Inhibition of T cell proliferation and IFNγ secretion by flCTLA-4 and liCTLA-4. TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells were infected with retrovirus expressing flCTLA-4 or liCTLA-4. Sorted GFP$^+$ cells were activated with anti-CD3 at 1 μg/ml and mitomycon-treated APCs consisting of syngeneic spleen cells depleted of CD4 and CD8 cells. TKO (B7.1, B7.2, CTLA-4$^{-/-}$) T cells and DKO (B7.1, B7.2) T cells infected with empty retrovirus (RV) were used as controls for CTLA-4 function.

(C) Mean ΔCPM in a proliferation assay as detected by 3[H] thymidine incorporation where ΔCPM is the difference in CPM obtained by subtracting CPM values of cells treated with media alone from that of CPM values of cells activated with anti-CD3.

(D) IFNγ production expressed as pg/ml in the supernatants of proliferation experiments as detected by cytokine ELISA in the supernatants. Data represent pooled values from two independent experiments. Error bars indicate standard error of mean.

(E) Proliferation of TKO (CTLA-4, B7.1, B7.2$^{-/-}$) cells infected with retrovirus expressing flCTLA-4, liCTLA-4, or retrovirus alone with PMA/ionomycin. Proliferative responses were measured by ^{3}H incorporation in triplicate wells and expressed as ΔCPM. Data in figure represent pooled values from two independent experiments. Error bars indicate standard error of mean.

encoding liCTLA-4, which completely lacks the extracellular IgV domain required for binding with B7-1 and B7-2 molecules, inhibited T cell proliferation and IFNγ secretion (Figures 3C and 3D) more efficiently than flCTLA-4. This implies that the extracellular domain of flCTLA-4 that sequesters B7 molecules may not be essential for the negative regulation of T cells. The cytoplasmic domain alone, possessing signaling capabilities, may be sufficient, if not more effective than flCTLA to inhibit T cells.

Ligation of TcR/CD3 and the extracellular domain of CTLA-4 are prerequisite for the inhibitory function of

CTLA-4. However, the liCTLA-4, which lacks a major portion of the extracellular domain, still inhibited T cell responses. This raised the issue of whether mitogenic signals that bypass proximal TcR signals could induce inhibitory signals by liCTLA-4. To test this, the same population of sorted GFP$^+$ cells that expressed fl or liCTLA-4 (Figures 3C and 3D) was stimulated with a combination of PMA/ionomycin. We found that sorted GFP$^+$ T cells expressing flCTLA-4 or liCTLA-4 showed comparable T cell responses as measured by thymidine incorporation (Figure 3E). Since T cell activation with PMA/ionomycin bypasses the requirement for the TcR it is possible that TcR crosslinking is essential for the negative regulation mediated by both flCTLA-4 and liCTLA-4 and is specific to membrane proximal signals mediated by TcR crosslinking.

It has been demonstrated that flCTLA-4 associates with the TcRζ chain within the immunological synapse (Chikuma et al., 2003; Egen and Allison, 2002). This association may recruit negative regulators of T cell activation, like the phosphatase SHP-2, into the synapse. To determine if the liCTLA-4 associates with the TCRζ and recruits SHP2, lysates from purified normal CD3$^+$ T cells were prepared from spleen cells obtained from unimmunized C57Bl/6 mice or from in vivo activated T cells obtained from mice injected intravenously 30 min previously with anti-CD3/CD28 antibodies (Marengere, 1996). Since there are no antibodies specific for liCTLA-4, in order to remove the flCTLA-4 the lysates were first immunoprecipitated (IP-1) with an anti-CTLA-4 antibody, 4F10 (Walunas, 1996), that binds to the extracellular IgV-like domain of flCTLA-4 (I-P1, lanes 1 and 2 in Figure 4A). The lysates depleted of flCTLA-4 were then subjected to a second immunoprecipitation for liCTLA-4 using C-19 antibody, specific for the cytoplasmic domain of CTLA-4 (I-P2, lanes 3 and 4 in Figure 4A). When I-P1 and I-P2 were probed with C-19 antibody on a Western blot, the anti-CTLA-4 4F10 antibody-derived I-P1 (which only binds to flCTLA-4) also showed the presence of liCTLA-4, suggesting that the flCTLA-4 and liCTLA-4 may associate in both resting and (to a lesser extent) activated T cells (Figure 4A, lanes 1 and 2). However, even after immunoprecipitation with the anti-flCTLA-4 4F10 antibody, the lysates from I-P2 still contained a pool of liCTLA-4 that existed independently of flCTLA-4. The flCTLA-4 immunoprecipitates that also contained associated liCTLA-4 could associate with the TcRζ and SHP-2 in both resting and activated T cells. However, in I-P2, a pool of liCTLA-4 found independent of flCTLA-4 contained the TcRζ chain, but no detectable SHP-2.

The above data suggest that both flCTLA-4 and liCTLA-4 can associate with TcRζ. However, only the complex involving flCTLA-4, liCTLA-4, and TcRζ could interact with SHP2. It was not clear from our data whether flCTLA-4 and liCTLA-4 when expressed independently would result in TcRζ association and SHP2 recruitment. To address this we utilized T cells from the TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice in which flCTLA-4 or liCTLA-4 were independently expressed by retroviral infection. Since the TKO T cells lack B7 molecules, resting T cells from TKO mice are physiologically naive compared to T cells obtained ex vivo from CTLA-4$^{-/-}$ mice. Hence activation-dependent association of either isoforms with the TcRζ chain can be more stringently analyzed in this system than in CTLA-4$^{-/-}$ T cells. In order to scale up the percentage of retrovirally infected CD4 cells for signaling work, we adopted an alternative strategy of infection wherin TKO T cells were activated with PMA/ionomycin and infected with the retrovirus in presence of retronectin. This approach enhanced the level of virally infected T cells to 60% as assessed by flow cytometric analysis for GFP expression (Figure 4B). Lysates were prepared from resting or activated TKO T cells infected with retrovirus encoding liCTLA-4 or flCTLA-4. CTLA-4 immunoprecipitates (using a C-19 anti-CTLA-4 antibody that recognizes both forms of CTLA-4) were blotted independently with the anti-TcRζ chain antibody and SHP2 antibody to examine CTLA-4/TcRζ association. In this system, we found an activation-dependent association of both liCTLA-4 and flCTLA-4 with the TcRζ (Figure 4Ci, lower panel). However, unlike our observation in normal T cells (Figure 4A, lanes 1 and 2), when liCTLA-4 and flCTLA-4 were expressed independently in CTLA-4$^{-/-}$ T cells, both molecules failed to interact with SHP2 in either resting or activated T cells (Figure 4Ci, upper panel). Thus, one could speculate that the inhibitory signaling complex formed by the association between the flCTLA-4, TcRζ, and SHP2 seen in normal T cells requires the coexpression of both flCTLA-4 and liCTLA-4.

It has been shown previously that the interaction of TcR and CTLA-4 results in SHP2 recruitment and dephosphorylation of the TcRζ chain to inhibit T cell responses (Lee et al., 1998). Our data showed that CTLA-4$^{-/-}$ T cells expressing either flCTLA-4 or liCTLA-4 alone were capable of inhibiting T cell responses. This seemed to be independent of SHP2 recruitment since flCTLA-4 and liCTLA-4 when expressed independently in CTLA-4$^{-/-}$ T cells interacted with TcRζ but failed to recruit SHP2. Hence it is possible that flCTLA-4 and liCTLA-4 can bring about dephosphorylation of the TcRζ even in the absence of SHP2 recruitment. To test this possibility, we examined the phosphorylation status of TcRζ in TKO T cells infected with vector alone, liCTLA-4, or flCTLA-4. In resting TKO T cells infected with vector alone, liCTLA-4, or flCTLA-4, we did not observe phosphorylation of the TcRζ. But upon CD3 crosslinking of TKO T cells infected with the vector alone, maximal phosphorylation of the TcRζ chain was achieved, indicated by the appearance of a phosphotyrosine band of p22. Infection of TKO cells with flCTLA-4 decreased the extent of TcRζ tyrosine phosphorylation, such that the bands corresponding to TcRζ now were of a lower molecular mass (p15, p18). Strikingly, in TKO T cells infected with liCTLA-4, no tyrosine phosphorylation of the TcRζ chain was observed (Figure 4Cii). These data demonstrate that both flCTLA-4 and liCTLA-4 can mediate dephosphorylation of the TcRζ chain even in the absence of SHP2 recruitment, suggesting that alternative negative signaling phosphatases may associate with the TcRζ/CTLA-4 complex. Second, the total ablation of TcRζ phosphorylation in liCTLA-4-infected TKO T cells suggests that even in the absence of B7 ligation, liCTLA-4 is more potent than flCTLA-4 in preventing phosphorylation of TcRζ chain. It should be noted that our conclusions are drawn from an overexpression system where the expression of either molecule is calcu-

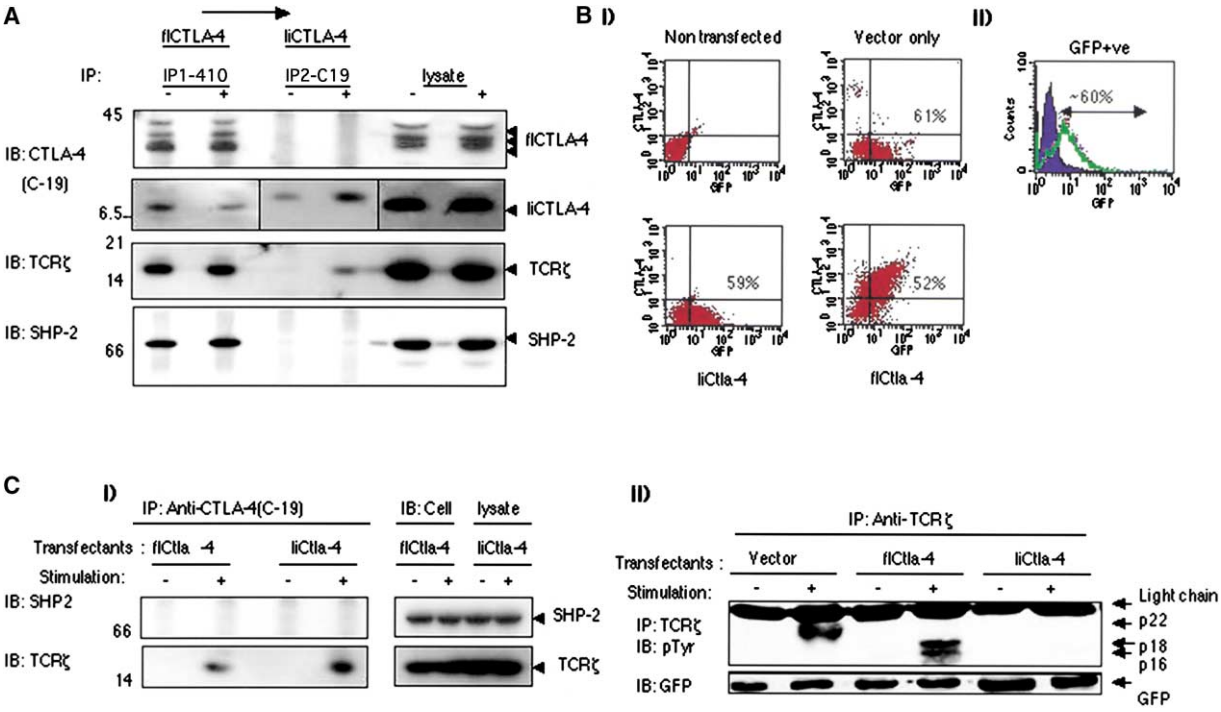

Figure 4. Signaling Mechanisms by which liCTLA-4 Inhibits T Cell Responses

(A) Association of liCTLA-4 and flCTLA-4 with the TcRζ chain and SHP2. Detergent lysates were prepared from 40×10^6 resting (-) or an equal number of in vivo activated (+) $CD3^+$ enriched T cells. The lysates were sequentially immunoprecipitated with an antibody to the extracellular (N-terminal) domain of flCTLA-4 (4F10) followed by an antibody to the C-terminal cytoplasmic domain of both CTLA-4 (C-19). Both immunoprecipitates and lysates were separated on a 10%–16% gradient gel and transferred to PVDF. The top half of the membrane, which was cut between 66 and 45 Kd, was probed with anti-SHP-2. The bottom half of the membrane was first probed with anti-CTLA-4 (C-19), detected with secondary rabbit anti-goat-HRP (anti-C-terminal blot, middle panel). The same membrane was then probed without stripping with an anti-TcRζ followed by anti-rabbit-HRP. The anti-rabbit-HRP further amplified the signal from the CTLA-4 isoforms and it is these amplified bands that are shown in the flCTLA-4 blot and the outer panels of the liCTLA-4 blot. Data are representative of three independent experiments.

(B) (i) Flow cytometric analysis of TKO (CTLA-4, B7.1, $B7.2^{-/-}$) T cells infected with the parental retrovirus (Vector) or retrovirus expressing CTLA-4 isoforms, flCTLA-4, or liCTLA-4 as monitored by GFP expression. (ii) Comparable GFP expression in infected TKO T cells. Shaded histogram indicates nontransfected control cells.

(C) (i) Activation-dependent association of flCTLA-4 and liCTLA-4 with the TCRζ chain and SHP2. TKO (CTLA-4, B7.1, $B7.2^{-/-}$) T cells were activated with PMA/ionomycin for 24 hr. Activated cells were infected with pGCIRES retrovirus expressing flCTLA-4, liCTLA-4, or control vector. Infected cells expressing GFP were rested in IL-2 medium and subsequently mixed with purified mitomycin-treated antigen-presenting cells in a ratio of 1:10. The mixture of T cells and APCs were incubated with anti-CD3 for 15 min on ice and then warmed to 37°C for 5 min. Detergent lysates were prepared from these cells (20×10^6/point), and flCTLA-4 or liCTLA-4 was immunoprecipitated using an antibody to the cytoplasmic domain (C-19). Immunoprecipitates were immunoblotted for associated SHP2 (top panel) and TcRζ chain (bottom panel). 2×10^6 lysates from the same pool of cells were immunoblotted with anti-TcRζ and SHP2 as a loading control (right panel). Data are representative of four independent experiments. (ii) liCTLA-4 is more effective than flCTLA-4 in inhibiting TcRζ phosphorylation. TKO (CTLA-4, B7.1, $B7.2^{-/-}$) T cells were infected with retrovirus expressing flCTLA-4, liCTLA-4, or control vector. Infected T cells expressing GFP were mixed with mitomycin C-treated purified APCs in a ratio of 1:10 and activated with anti-CD3 for 5 min. TCRζ was immunoprecipitated from lysates of 10×10^6 cells. Immunoprecipitates were immunoblotted with anti-phosphotyrosine antibody. Blots were stripped and reprobed with anti-GFP antibody to demonstrate equivalent loading. Data are representative of four independent experiments.

lated and compared based on GFP expression. Nevertheless, based on this data one could conclude that the liCTLA-4 is a potent inhibitory molecule with an ability to regulate proximal T cell signaling events as demonstrated by its ability to dephosphorylate the TcRζ in activated T cells.

We next wished to study the significance of our finding in T cells from the diabetes-resistant NOD.B10 *Idd5.1* congenic strain. The diabetes susceptibility locus defined by *Idd5.1* on mouse chromosome 1 is a small genetic interval of <1.5 cM of DNA that includes only the CTLA-4 and ICOS costimulatory molecules (Hill et al., 2000). Recent evidence suggests that this linkage may be due to a SNP in exon 2 that regulates expression of liCTLA-4 (Ueda et al., 2003). Since mRNA expression of flCTLA-4 is low in naive T cells and increased in activated (Alegre et al., 1996) and memory/regulatory T cells (Metz et al., 1998; Read et al., 2000), we wanted to determine relative expression of mRNA transcripts for flCTLA-4 versus liCTLA-4 in naive versus memory/regulatory T cells. Furthermore, we wanted to determine whether the expression differs between autoimmune susceptible and resistant strains of mice. For this purpose, we compared the expression of flCTLA-4 and liCTLA-4 in naive and memory/regulatory T cells of two autoimmune susceptible (NOD and SJL, which encode the G186 SNP in exon 2 of CTLA-4) and resistant (B6.*H2g7* and B10.S, which encode the A186 SNP in

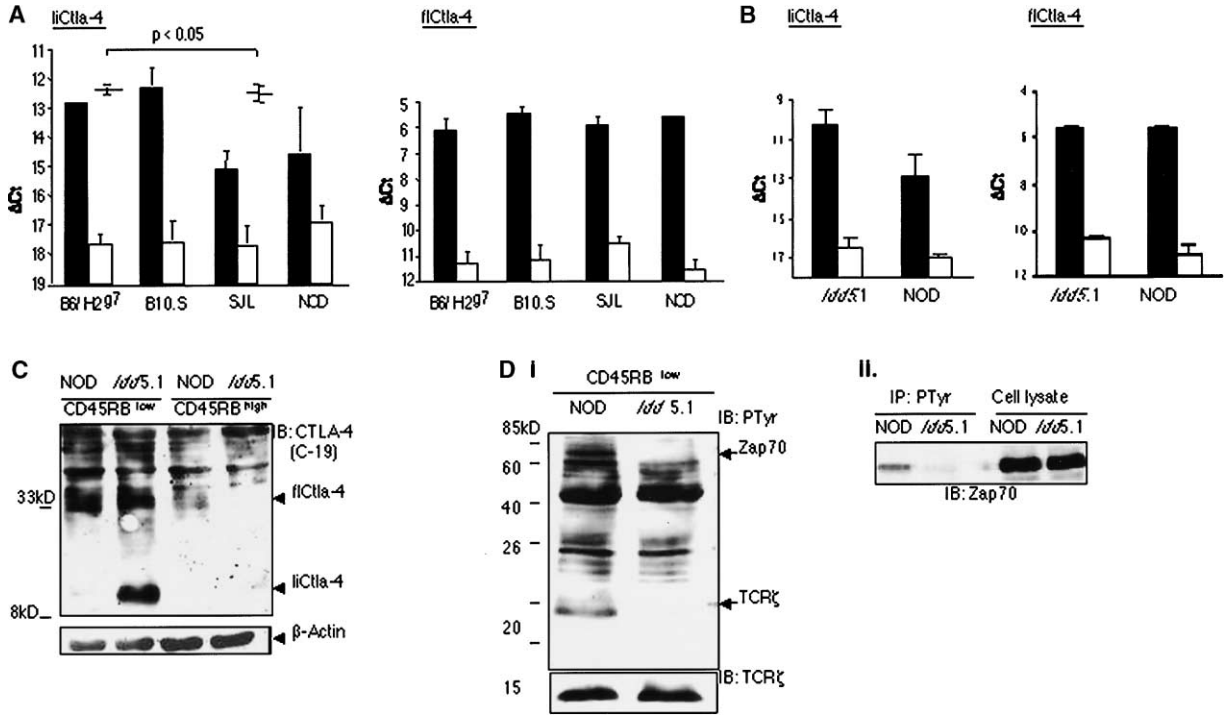

Figure 5. liCtla-4 Expression Is Higher in CD45Rb^{low} T Cells

(A) Expression of liCTLA-4 and flCTLA-4 in fractionated CD4$^+$CD45RB^{low} and CD4$^+$CD45RB^{high} cells of different mouse strains was examined by real-time RT-PCR and represented as ΔCt values that were calculated by Ct values (threshold cycle of amplification) of triplicates normalized to GAPDH as described in the Experimental Procedures. mRNA expression in fractionated ex vivo CD4$^+$CD45RB^{low} (shaded bars) and CD4$^+$CD45RB^{high} (open bars) cells of autoimmune susceptible (SJL/J, NOD) and resistant strains (B10.S, C57Bl/6^{g7}).

(B) mRNA expression of liCTLA-4 and flCTLA-4 in fractionated ex vivo CD4$^+$CD45RB^{low} (shaded bars) and CD4$^+$CD45RB^{high} (open bars) cells from NOD. B10 *Idd5.1* congenic and wild-type NOD mice.

(C) Increased protein expression of liCTLA-4 in the diabetes resistant NOD. B10 *Idd5.1* congenic mice. Cell lysates were obtained from CD4$^+$CD45RB^{low} and CD4$^+$CD45RB^{high} cells sorted ex vivo from diabetes susceptible NOD and resistant NOD.B10 *Idd5.1* mice and resolved on a 12% reducing gel. Expression of liCTLA-4 and flCTLA-4 was examined by Western blotting using the C-19 antibody that recognizes both isoforms of CTLA-4. Data are representative of three independent experiments.

(D) (i) Reduced TcRζ and ZAP70 phosphorylation in CD45RB^{low} cells of NOD.B10 *Idd5.1* congenic mice. Cell lysates were prepared from 3 × 10^6 CD4$^+$CD45RB^{low} cells fractionated ex vivo from diabetes susceptible NOD and resistant NOD.B10 *Idd5.1* mice and resolved on a 12% reducing gel. Sequential blotting was done using anti-pTyr, anti-CD3ζ, and anti-ZAP70 antibody. Data are representative of four independent experiments. (ii) Comparison of the phosphorylation status of ZAP-70 in CD45RB^{low} cells of NOD.B10 *Idd5*.1 congenic mice. Cell lysates were prepared from 10–12 × 10^6 CD4$^+$CD45RB^{low} cells fractionated ex vivo from diabetes susceptible NOD and resistant NOD.B10 *Idd5.1* mice. Lysates were immunoprecipitated using anti-phosphotyrosine antibody and immunoblotted using anti-ZAP antibody. Data are representative of four independent experiments.

exon 2 of CTLA-4) strains of mice. Peripheral T cells were sorted into naive (CD4$^+$CD45RB^{high}) and memory/regulatory (CD4$^+$CD45RB^{low}) T cells based on the CD45RB phenotype and analyzed using real-time (Taq-Man) RT-PCR to determine mRNA levels for flCTLA-4 and liCTLA-4. As expected, expression of both CTLA-4 isoforms was very low in the naive CD4$^+$CD45RB^{high} T cells (Figure 5A). However, there was a significantly higher expression of both flCtla-4 and liCTLA-4 in the CD4$^+$CD45RB^{low} cells. On comparing the mRNA transcript levels of liCTLA-4 in the CD4$^+$CD45RB^{low} cells between the autoimmune resistant strains and autoimmune susceptible strains, we found that the autoimmune resistant strains expressed a much higher level of liCTLA-4 than the autoimmune susceptible strains. Figure 5A shows that the relative amplification of the liCTLA-4 mRNA with respect to GAPDH, a housekeeping gene, proceeded earlier in the autoimmune resistant strains than the susceptible strains. This data, when expressed as a ratio of mRNA transcript levels of liCTLA-4 to that of GAPDH, showed that the level of liCTLA-4 mRNA transcripts in the CD4$^+$CD45RB^{low} cells of autoimmune resistant strains was approximately four times higher than that expressed in autoimmune susceptible strains (Figure 5A). We did not observe any significant difference in the level of mRNA transcript for flCTLA-4 among the four different strains of mice, in either the CD45RB^{low} and CD45RB^{high} T cell populations (Figure 5A). This differential expression of flCTLA-4 and liCTLA-4 was more evident in memory/regulatory T cells of NOD mice and NOD *Idd5.1* congenic mice. The resistance locus in the NOD.B10 *Idd5.1* congenic strain has been mapped to an ~200 Mb genomic interval and carries the genes encoding for CTLA-4 and ICOS from B10 mice on NOD background (L.S.W. et al., unpublished data). Increased disease risk was correlated with differential mRNA levels of liCTLA-4 (Ueda et al., 2003). When CD45RB^{high} or CD45RB^{low} CD4$^+$ cells from the NOD and NOD.B10

Idd5.1 strains were analyzed for expression of flCTLA-4 or liCTLA-4 by real-time Taqman RT-PCR, there was a significantly higher expression of liCTLA-4 mRNA in the cells from the NOD.B10 *Idd5.1* congenic mice (Figure 5B). But there was no difference in the level of expression of flCTLA-4. The basal differences seen in the liCTLA-4 mRNA expression in the different strains were also evident at the level of liCTLA-4 protein expression (data not shown). As seen in Figure 5C, memory/regulatory $CD4^+CD45RB^{low}$ T cells showed a much higher level of liCTLA-4 protein expression than that of naive $CD4^+CD45RB^{high}$ T cells in NOD.B10 *Idd5.1* strain compared to the wild-type NOD strain.

The increased expression of liCTLA-4 in the $CD4^+CD45RB^{low}$ may restrict clonal expansion of memory T cells. This could be best tested in a comparison between T cells from NOD and NOD B10. *Idd5.1* congenic in which T cell function is primarily influenced by the difference in the levels of liCTLA-4 expression and not by other background genes. Since our data suggest that liCTLA-4 binds and inhibits phosphorylation of the TcRζ chain, we reasoned that memory/regulatory ($CD4^+CD45RB^{low}$) T cells of NOD or NOD.B10 *Idd5.1* mice that differ in the levels of liCTLA-4 expression may also differ in the extent of TcRζ phosphorylation. To address whether the naturally occurring enhanced expression of liCTLA-4 in NOD.B10 *Idd5.1* T cells would alter the phosphoryla-tion status of the TcRζ chain and subsequent signal-ing events, we analyzed lysates prepared from $CD4^+CD45RB^{low}$ T cells from NOD or NOD.B10 *Idd5.1* mice. Immunoblotting with an anti-phosphotyrosine antibody demonstrated that bands corresponding to TcRζ and ZAP-70 (Figures 5Di and 5Dii) were not phosphorylated in the $CD4^+CD45RB^{low}$ T cells from NOD.B10 *Idd5.1* mice as compared to the $CD4^+CD45RB^{low}$ T cells from NOD mice that were strongly phosphorylated. This altered pattern of phosphorylation in ex vivo isolated memory/regulatory T cells from NOD.B10 *Idd5.1* mice that express higher levels of liCTLA-4 as compared to the $CD4^+CD45RB^{low}$ from NOD mice suggests that the liCTLA-4 can indeed regulate the activation status of these cells in vivo in the diabetes-prone NOD mice.

Discussion

CTLA-4 plays a critical role in maintaining peripheral tolerance and preventing autoimmunity. The genetic locus encoding the CTLA-4 gene has been implicated in multiple autoimmune diseases in both mouse and human. In a recent study, genetic variations in the expression levels of splice variants of the CTLA-4 gene were associated with susceptibility to autoimmune diseases (Ueda et al., 2003). In mice, it was shown that subtle inherited variations in the mRNA levels of a novel splice variant called liCTLA-4 were responsible for diabetes susceptibility in the NOD mice. However, whether this variation in the expression of liCTLA-4 affects T cell function and thus accounts for the difference in genetic susceptibility was not analyzed in that study. The mRNA encoding liCTLA-4 was found to lack exon 2, which encodes for most of the extracellular domain of flCTLA-4, essential for interaction with its ligand, B7.1 and B7.2. Since flCTLA-4 crosslinking with B7 is considered essential for negative signaling, it was important to determine the function of this naturally occurring, genetically regulated isoform of CTLA-4 that lacks B7 binding domain.

Our study shows that the liCTLA-4 is expressed as a protein in T cells and functions to downregulate T cell responses. However, the expression kinetics of the liCTLA-4 was different from the flCTLA-4. Whereas flCTLA-4 was upregulated in T cells following activation, the liCTLA-4 was expressed at high levels in resting T cells and rapidly downregulated early during T cell activation. We found that the major expression of liCTLA-4, at the mRNA and protein level, was in the $CD4^+CD45RB^{low}$ T cells, suggesting that expression of liCTLA-4 observed in resting T cell populations must come from the memory/regulatory T cell population. The mechanism, by which resting/memory T cells are kept under check and prevented from activation by chronic exposure to weak stimulation by circulating crossreactive self-antigens, is not known. Memory ($CD4^+CD45RB^{low}$) T cells, which have low threshold requirements for activation, have been shown to respond to weak TcRζ signals even in the absence of essential costimulatory signals delivered via CD28 (Metz et al., 1998). We postulate that high expression of liCTLA-4 in the memory/regulatory T cells prevents them from activation following exposure to weak antigens. Stronger stimuli, however, induce downregulation of liCTLA-4, permitting T cells to progress into mitosis resulting in T cell expansion. On the contrary, it has been shown that flCTLA-4 inhibits activation of T cells mediated by high-affinity antigens and strong activation signals (Egen and Allison, 2002; Linsley et al., 1996). Hence it is possible that, whereas liCTLA-4 functions to prevent activation of memory/effector T cells to weak stimuli, flCTLA-4 inhibits progression of activated T cells when stimulated by high-affinity antigens. This speculation is consistent with our data showing that liCTLA-4 is constitutively expressed in memory/effector T cells and downregulated early following T cell activation, whereas flCTLA-4 is expressed in activated T cells.

Though the liCTLA-4 lacks the extracellular B7 binding domain, it can still function as a negative regulator of T cell responses. This suggests that the cytoplasmic tail of CTLA-4, independent of the extracellular B7 binding domain, is capable of delivering a negative signal. Inhibition by flCTLA-4 has been proposed to be achieved by two different mechanisms: delivery of an inhibitory signal by the cytoplasmic tail and sequestration of B7 by the extracellular domain (Carreno et al., 2000; Nakaseko et al., 1999). A combination of both these events must be employed by flCTLA-4 to exert its full inhibitory function. It has been shown that mice expressing a mutant CTLA-4 lacking the cytoplasmic tail on the $CTLA\text{-}4^{-/-}$ background could partially rescue the multiorgan lymphocytic infiltration characteristic of $CTLA\text{-}4^{-/-}$ mice. Similarly, $CTLA\text{-}4^{-/-}$ mice expressing a Y201V transgene, where the Tyr residue in the cytoplasmic tail, critical for trafficking and signaling, was replaced by a Valine, showed a complete rescue from lymphoproliferation and autoimmunity (Masteller et al., 2000). Both these studies suggest that the extracellular domain of CTLA-4 can independently mediate T cell inhibition by seques-

tration of B7 away from CD28 and thus prevent induction of positive costimulation into T cells. Our study shows that, even in the absence of an extracellular domain, the cytoplasmic domain of liCTLA-4 alone is sufficient to deliver a negative signal similar to that delivered by flCTLA-4.

The molecular mechanism of T cell inhibition by flCTLA-4 involves the interaction of TcR and CTLA-4 with SHP2 recruitment and dephosphorylation of the TcRζ chain (Lee et al., 1998). When we independently expressed either the flCTLA-4 or the liCTLA-4 in CTLA-4$^{-/-}$ T cells, both liCTLA-4 and flCTLA-4 could independently associate with the TcRζ chain. However, the lack of phosphorylation of the TcRζ chain in this situation was found to be SHP2 independent. But in normal T cells from wild-type mice, which coexpress both isoforms, liCTLA-4 associated with flCTLA-4, and it is only this complex that could concentrate the key inhibitory phosphatase, SHP-2, in both resting and activated T cells. The association of liCTLA-4 and flCTLA-4 appears to be quite stable, since we could detect liCTLA-4 in the flCTLA-4 immunoprecipitates after extensive washing, whereas the SHP2 was lost from the flCTLA-4 immunprecipitates under these conditions (data not shown). This observation might in part explain why many studies have not been able to see the binding of SHP-2 with the flCTLA-4. Our data suggest that interaction of SHP2 with TcRζ is brought about by the heterodimerization of liCTLA-4 and flCTLA-4, since a pool of liCTLA-4 found in normal T cells and not associated with flCTLA-4 failed to bring SHP2 into the liCTLA-4-TcRζ complex. Although this requires further analysis, our data nevertheless clearly demonstrate that when liCTLA-4 and flCTLA-4 are expressed together in T cells they can interact with TCRζ and SHP2 but when expressed independently they directly associate with TcRζ chain and prevent its tyrosine phosphorylation independent of SHP-2 binding. This lack of phosphorylation of the TcR may be due to a physical blockade of kinases that phosphorylate the TcR as opposed to dephosphorylation by inhibitory phosphatases. This is not simply due to in vitro overexpression of liCTLA-4 in the infected cells since natural overexpression of liCTLA-4 in the diabetes resistant congenic strain (NOD.B10 *Idd5.1*) also resulted in the downregulation of tyrosine phosphorylation of the TcRζ chain.

The method of inhibition by liCTLA-4 may provide a novel molecular mechanism by which resting/memory T cells are kept in check from activation. If indeed liCTLA-4 expressed at high levels in memory/activated T cells renders them nonresponsive to crossreactive low-affinity self-antigens then liCTLA-4 provides an important checkpoint for maintenance of self-tolerance in the periphery. Alternatively, since the CD4^{+}CD45RB^{low} population is known to contain CD4^{+}CD45RB^{low}CD25^{high} T regulatory cells, genetically controlled levels of liCTLA-4 in CD4^{+}CD45RB^{low} T cells could influence the development or activity of these cells. Thus, increased expression of liCTLA-4 in the CD4^{+}CD45RB^{low} cells in autoimmune disease resistant mice may result in enhanced regulatory T cell function. It will be interesting to see whether overexpression of liCTLA-4 in a transgenic system will generate more CD4^{+}, CD25$_{+}$ regulatory T cells like what was seen with the overexpression of Fox-P3 (Khattri et al., 2003).

In summary, our data provide evidence for an inhibitory role for a novel isoform of CTLA-4, which attenuates T cell responses even though it lacks an extracellular domain required for interaction with B7 molecules. However, its expression kinetics and mechanism of negative regulation seem to be distinct from that of flCTLA-4. Like liCTLA-4, one of the isoforms of human CD28 called CD28i was also found to lack exon 2 (Hanawa et al., 2002). Reminiscent of the biological activity of liCTLA-4, when CD28i was transfected in Jurkat T cells T cell activation and IL-2 secretion were enhanced. Genetic evidence in human autoimmune disease suggests that protection from diabetes is not due to a structural polymorphism in the coding sequence of CTLA-4 gene but instead is correlated with the expression level of an alternatively spliced form of CTLA-4 (sCTLA-4). The causative variant associated with diabetes susceptibility is mapped to a noncoding 6.1 kb 3′ region of CTLA-4 that determines levels of the sCLTA-4 transcript (Ueda et al., 2003). These data together with the functional data presented here suggest that genetic variation of the expression of alternatively spliced forms of CTLA-4 contribute to the genetic control of autoimmunity in both mice and humans.

Experimental Procedures

Mice

6- to 8-week-old female C57BL/6 and NOD/LtJ mice were obtained from Jackson laboratories (Bar Harbor). In addition, 6- to 8-week-old NOD.B10 *Idd5.1* R46 mice were obtained from Taconic (Germantown, New York). BALB/c DO11.10 TCR transgenic mice were bred on the RAG2$^{-/-}$ background and maintained at our facility. BALB/c triple knockout mice (TKO) lacking B7.1, B7.2, CTLA-4$^{-/-}$, and BALB/c double knockout mice (DKO) lacking B7.1/ B7.2$^{-/-}$ (Mandelbrot et al., 1999) mice were generated by Arlene Sharpe (Harvard Medical School, Boston, MA).

mRNA Isolation and RT-PCR

Total mRNA was isolated from spleen cells of C57BL/6 mice activated with 1 μg/ml anti-CD3 (Pharmingen) using the Qiagen mRNAEasy kit (Qiagen). cDNA from 5 μg total mRNA was prepared by Oligo dT priming using the Superscript reverse transcriptase kit (Invitrogen Life Technologies). RT-PCR amplification of CTLA-4 isoforms was done using the Exon 1 5′ primer CCGCTCGAGTTGGG TTTTACTCTACTCCCTGA and Exon 4 3′ primer ACGCGTCGACTCC TTCTTCTTCATAAACGGC. Amplified products were cloned into TOPO 2.1 vector. A Exon 1/3 junctional primer was used to specifically amplify liCTLA-4 from mRNA synthesized from activated spleen cells. The forward exon1/exon3 primer was TCTCGAAGATCCAG and reverse primer in exon 4 was TCCTTCTTCTTCATAAACGGC. Cloned products were sequenced to determine and confirm the sequence of flCTLA-4 and liCTLA-4.

Construction of Expression Vectors and Transfection of HEK293T Mammalian Cells

liCTLA-4 was directionally cloned in the NotI/SalI site within the multiple cloning site of pCMV-Tag5C. The stop codon in liCTLA-4 was mutated using: 5′TTTATTCCCATCAACGGAAAGGCCGTTTAT GAAGAAGAAGGA and a complimentary 3′ primer using the Quickchange site-directed mutagenesis kit (Stratagene). Insertion of a 10 amino acid MYC site between the signal sequence and the extracellular domain was achieved by first cloning the cDNA encoding the liCTLA-4 in the Xho1 site of pCMVTag2C vector. Then using the primers 5′ GTCTTCGAAGAT***GCCGCCATGGAGCAGAAACTCATC TCTGAA***GAGGATCTGCCAGAACCATGCCCG and a 3′ complimentary reverse primer, a MYC site was inserted in a site-directed muta-

genis reaction using the Quickchange site-directed mutagenesis kit (Stratagene). Sequence in italics indicates the MYC site. For intracellular localization studies, liCTLA-4 in which the stop codon had been mutated as described above was cloned into the EcoRI site of pDsRedExpress-N1 vector (CLONTECH). Stop codon for flCTLA-4 was mutated as described for liCTLA-4 and cloned in the XhoI site of pEGFP-N3. flCTLA-4 lacking the cytoplasmic tail was amplified using the forward 5′ primer CCGCTCGAGTTGGGTTTTAC TCTACTCCCTGA and a reverse primer CCGCTCGAGCAAGCTAAC TGCGACAAGGATCCA. The amplified product was later cloned into the XhoI site of pEGFP-N3 (CLONTECH). For retroviral expression, cDNA of flCTLA-4 and liCTLA-4 were cloned into the Xho1 restriction site of the pGCIRES retroviral vector developed by Garry Nolan (Costa et al., 2000). Cloned products were confirmed for presence and orientation of inserts by sequencing at the Brigham and Women's Hospital sequencing core.

Flourescent microscopy and confocal studies. Flourescent tagged mammalian expression cloning vectors encoding liCTLA-4, flCTLA-4, or flCTLA-4 tailless were transiently transfected in HEK293T cells using SUPERFECT (Invitrogen) following the manufacturer's protocol. Transfected cells were fixed in 4% paraformaldehyde and mounted on a glass slide. Image capture was done with a confocal microscope (Carl Zeiss, Inc) and analyzed with the LSM 510 software (Carl Zeiss, Inc.; Microsoft). Comparision of intracellular localization in HEK293 cotransfectants expressing liCTLA-4 and flCTLA-4 were studied using a Zeiss Axioskop 2 plus, mounted with a Plan-Apo chromat 63× objective and OSRAM HBO Mercury Short Arc Lamp.

Cell Preparation and Retroviral Infections

Ecotropic packaging cells (Phoenix-E) were cultured in DMEM complete medium (DMEM-10% Fetal Bovine Serum, GIBCO-BRL) and transfected with retroviral constructs using Lipofectamine (Life Technologies). Retrovirus-containing cell supernatant was collected 48 and 72 hr after transfection and used to infect in vitro activated C57BL/6/TKO (B7.1, B7.2, CTLA-4$^{-/-}$) and C57BL/6/DKO (B7.1, B7.2) T cells as described previously (Costa et al., 2000). Viral supernatants expressing liCTLA-4, flCTLA-4, or empty vector were titrated for infectivity based on their ability to infect NIH3T3 cells. Titrated supernatants were then used to infect CD3$^+$ T cells from C57BL/6/TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice activated with plate- bound anti-CD3 and anti-CD28 (2 μg/ml each) in the presence of 8 μg/ml polybrene. 24 hr later cells were reinfected by the same protocol for another 12 hr, after which cells were rested in IL2 medium for 72 hr before analysis. Using this approach, we could routinely transduce 10%–20% of activated T cells with the retroviral vector. For biochemistry, CD3$^+$ T cells from C57BL/6/TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice were activated with PMA/ionomycin at 1 μg/ml PMA and 50 nM ionomycin for 24 hr in DMEM media containing 10% fetal calf serum. Activated cells were infected in two rounds with the retroviral constructs by plating cells on non-tissue culture-treated Retronectin coated plates for 12 hr. Following this, cells were rested for 96 hr in DMEM clone medium containing IL2 for viral integration.

Proliferation and Cytokine Production

Proliferative responses were measured by culturing 3×10^4 sorted GFP$^+$ T cells in the presence of mitomycin C-treated, CD4 and CD8 depleted APCs with anti-CD3 (145-2C11, Pharmingen) for 48 hr or PMA/ionomycin at 1 μg/ml PMA and 50 nM ionomycin for 24 hr in DMEM media. Each concentration was set up in triplicate wells. After the indicated times cells were pulsed with 1 μCi of 3H$^+$ thymidine for 16 hr and then harvested for counting on a Beta plate scintillation counter (Wallac). Data are represented as mean CPM in triplicate wells. Culture supernatants were collected after 48 hr and analyzed for IFNγ by standard ELISA as described previously (Nicholson et al., 1995).

Flow Cytometric Analysis and Western Blotting

Surface expression of MYC on HEK293T cells transfected with MYC tagged liCTLA-4 was monitored by staining transfected cells with FITC-conjugated goat anti-MYC antibody (Santa Cruz). flCTLA-4 expression in T cells infected with retrovirus was detected using PE-anti-CTLA-4 antibody (4F10, Pharmingen). CD4 cells were enriched using an R&D T cell column (R&D Systems). Naive and memory T cells within the CD3 enriched T cell population of ex vivo harvested spleen cells were stained with FITC-anti-CD4 (Pharmingen) and PE-anti-CD45RB (Pharmingen). FACS analysis and sorting was performed on a Becton Dickinson FACS Calibur flow cytometer.

Cell lysates from 2×10^6 spleen cells of C57BL/6 mice activated with anti-CD3 (2 μg, 2C11, Pharmingen) and 2×10^6 GFP + ve retrovirus-infected TKO T cells were prepared using lysis buffer containing 1% NP-40, 150 mM NaCl, 25 mM Hepes [pH 7.5], 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Cell extracts were run on a 12% denaturing gel. Fractionated proteins were transferred on to a PVDF membrane (BioRad) and probed with specific antibodies to MYC (mouse anti-MYC antibody, Santa Cruz), CTLA-4 (Hamster anti-CTLA-4, 4F10, Pharmingen), or with goat anti-CTLA-4 antibody recognizing the cytoplasmic domain of CTLA-4 (C19, Santa Cruz). GFP expression was detected with an anti-GFP antibody (Monoclonal anti-GFP, 8369-1, Clontech). 2×10^6 CD4$^+$CD45RB^{high} or CD4$^+$CD45RB^{low} cells from 6- to 8-week-old SJL, B10, C57/Bl6, or NOD mice were lysed and subjected to immunoblot with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology). To detect the phosphorylation status of ZAP-70 in CD4$^+$CD45RB^{low} cells, lysates from 10–12 $\times$ 10^6 cells were lysed as detailed above. Cell lysates were incubated first with 10 μl of anti-phosphotyrosine antibody (4G10, Upstate Biotechnology) plus 20 μl protein A/G agarose for 1 hr at 4°C. The immunoprecipitates were washed three times with lysis buffer. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), blocked with 5% nonfat milk in TBST, immunoblotted with anti-ZAP-70 antibody (Santa Cruz Biotechnology, Inc.), and detected using HRPO-conjugated secondary anti-rabbit antibody (Pharmingen) followed by chemiluminesence (ECL).

For detection of CTLA-4 association with TCRζ and SHP-2, the cells were lysed for 15 min on ice in cold lysis buffer (1% NP-40, 150 mM NaCl, 25 mM Hepes [pH 7.5], 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Detergent extracts were clarified by centrifugation at 14,000 × g for 10 min at 4°C. The resulting supernatants were harvested and either used for immunoprecipitations or separated by electrophoresis through 10%–16% SDS/polyacrylamide gels (SDS-PAGE) (Protogel, National Diagnostics, Atlanta, GA). For immunoprecipitation, cellular extracts were incubated first with 10 μl of anti-CTLA-4 antibody recognizing the extracellular domain (4F10) plus 20 μl protein A agarose/20 μl protein G agarose for 1 hr at 4°C, after which the lysates were reimmunoprecipitated to 10 μl CTLA-4 antibody, specific for cytoplasmic tail of CTLA-4 (C-19) and 40 μl of protein G agarose for 2 more hr at 4°C. The 4F10/protein A/G beads were resuspended in sample buffer. The C19 CTLA-4 immunoprecipitates were washed three times with lysis buffer. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), blocked with 5% nonfat milk in TBST, immunoblotted with CTLA-4 antibody (C-19), polyclonal anti-TCRζ antisera (387, the generous gift of L. Samelson, NIH), or with anti-SHP-2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and detected by rabbit anti-goat-HRP or donkey anti-rabbit-HRP (Amersham) followed by enhanced chemiluminesence (ECL).

To analyze the induction of tyrosine phosphorylation upon TcR crosslinking, T cells from C57BL/6/TKO (B7.1, B7.2, CTLA-4$^{-/-}$) mice infected with control retrovirus or retrovirus expressing flCTLA-4 or liCTLA-4 were mixed with antigen-presenting cells as described previously in a ratio of 1:10 TKO T cells: APC. 10×10^6 cells were activated with anti-CD3ε (clone 145-2C11, Pharmingen) at 1 μg/ml for 10 min for anti-TCRζ immunoprecipitations. To analyze phosphorylation status, 5×10^6 cells were treated with 1 mM pervanadate for 2 min at 37C, lysed in lysis buffer for 30 min, and centrifuged for 10 min at 8000 g. Lysates were run on a 12% reducing gel and phosphorylated proteins detected using an anti-phosphotyrosine antibody (clone 4G10, Upstate Biotechnology) and HRPO-conjugated secondary anti-mouse antibody. Western blots were developed using an ECL kit (Amersham) and exposed to XAR film (Kodak).

Real-Time (TaqMan) RT-PCR

CD3 + ve T cells from 6- to 8-week-old NOD/LtJ, SJL, B10, C57/Bl6, and NOD.B10 *Idd5.1* R426 were enriched using T cell enrichment

columnn (R&D Systems). Cells were stained with CD4-FITC and CD45RB-PE and sorted for $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ expression. Total mRNA was isolated from ex vivo sorted $CD4^+CD45RB^{high}$ or $CD4^+CD45RB^{low}$ cells activated with 1 μg/ml anti-CD3 (Pharmingen) using the Qiagen mRNAEasy kit (Qiagen). mRNA (2–3 μg) was digested with DNase I (Gibco-Invitrogen) and transcribed to cDNA with oligo d(T) 16 primers and random hexamers using the TaqMan Reverse Transcription Reagents according to manufacturer's instructions (Applied Biosystems). Quantitative real-time polymerase chain reaction was performed on an ABI Prism 7700 Sequence Detection System (Perkin Elmer, Foster City, CA) using cDNA as template. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for sample normalization in a multiplex RT-PCR approach (TaqMan Rodent GAPDH Control Reagents, Applied Biosystems). The amplification followed the protocol of the TaqMan Gold RT-PCR kit. For detection of liCTLA-4 and full-length CTLA-4, as well as GAPDH transcripts, oligonucleotides were used at final concentrations of 300 nM for forward and reverse primers and 200 nM for the fluorogenic probes as follows:

flCTLA-4:
Forward: ACTCATGTACCCACCGCCATA
Reverse: GGGCATGGTTCTGGATCAAT
Probe: CATGGGCAACGGGACGCAGATTTAT
liCTLA-4:
Forward GCCTTTTGTAGCCCTGCTCA
Reverse TCAGAATCCGGGCATGGTT
Probe TTCTTTTCATCCCAGTCTTCTCTGAAGATCCA

ΔCt values were obtained by calculating Ct values of each well ($Ct^{liCTLA-4}$-Ct^{GAPDH}). ΔΔCt values were determined by normalization of average ΔCt values of triplicates to average ΔCt values of no template control (NTC) triplicates (ΔCt-ΔCt^{NTC}).

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